

Remarks

Applicants note with appreciation that the outstanding Office action withdraws the following rejections:

claims 1, 3-10, 12, 15, 16, and 18 under 35 U.S.C. §102(e) in view of Frondoza et al. (U.S. patent application number 2005/0147959);

claims 1, 3-7, 10, 12 and 16 under 35 U.S.C. §102(e) in view of Feygin et al. (U.S. patent number 6,315,957); and

claims 2, 11, 13, 14, and 17 under 35 U.S.C. §103(a) in view of Feygin et al. (U.S. patent number 6,315,957) in combination with Reeve et al. (International patent application number WO 91/12079) and Valkirs et al. (U.S. patent number 63,483,18).

Upon entry of this Response, claims 1, 3-16, and 18-20 are pending. No new matter has been added, and no new material presented that would necessitate an additional search on the part of the Examiner.

Prior to responding to the Office action, applicants believe it would be of convenience to the reader to review the subject matter of independent claims 1 and 12.

Claim 1 is directed to an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom for receiving and retaining the objects, in which the objects are at least one object selected from the group of affinity beads and one or more living organisms, in which the receptacle is permeable on the bottom and on a portion of at least one side to permit the liquid to flow through the receptacle to intermix with the objects when the receptacle is inserted into a vessel containing the liquid, with the proviso that a portion of at least one side is not permeable.

Claim 12 is directed to an apparatus for intermixing objects with a liquid including a plurality of receptacles, each receptacle having sides and a bottom for containing the objects, in which the objects are at least one object selected from the group of affinity beads and one or more living organisms, each of the receptacles is permeable on the bottom and on a portion of at least one side to permit the flow of the liquid therethrough, with the proviso that a portion of at least one side is not permeable, in which a vessel containing the solution, and means for repeatedly inserting the receptacles into the liquid in the vessel and then withdrawing the receptacle from the liquid to cause the liquid to first flow into the receptacle through the

permeable portion when the receptacle is inserted into the liquid and then to flow outwardly from the receptacle through the permeable portion when the receptacle is withdrawn from the liquid.

Claims comply with 35 U.S.C. §103(a)

The Office action on p. 3 ¶2 rejects claims 1, 3-16, and 18 under 35 U.S.C. §103(a) in view of Frondoza et al. (U.S. patent application number 2005/0147959, published July 7, 2005) in combination with Robinson et al. (U.S. patent number 6,890,740 issued May 10, 2005). Applicants respectfully traverse.

The ultimate determination of whether an invention would have been obvious under 35 U.S.C. §103(a) is a legal conclusion based on underlying findings of fact. *In re Kotzab*, 217 F.3d 1365, 1369 (Fed. Cir. 2000).

As a preliminary matter, the Supreme Court in *Graham v. John Deere*, 383 U.S. 1 provided an analytical construct to be used when determining whether claims are obvious under 35 U.S.C. §103(a) in view of prior art. One aspect of this analytical construct includes characterizing the prior art, as a background for a legal analysis.

Frondoza et al. (U.S. patent application number 2005/0147959, published July 7, 2005)

Frondoza shows a test system and method for making and using tissue analogs (Frondoza, Abstract). Frondoza is titled, “Tissue analogs for in vitro testing and method of use therefor”. Frondoza’s method involves the following steps: isolating cells from donor tissue; seeding the cells onto a particulate microcarrier bead; culturing the cells on microcarrier beads to achieve an expansion in the number of cells; and further culturing cell-particle aggregates to form a tissue analog (Ibid., Abstract).

Frondoza shows a two-component multi-well culture plate system for culturing of cells to form tissue analogs (Ibid., ¶[0051] and Fig. 1). One component is an insert having molded wells and an open bottom (Ibid., ¶[0051] and Fig. 1). The open bottom of each well is fitted with a porous screen that covers the bottom of each well (Ibid., ¶[0051] and Fig. 1). The other component is a standard microtiter plate (Ibid., ¶[0051] and Fig. 1), and the wells of the insert fit into the wells of the microtiter plate to form the two-component multi-well culture plate system (Ibid., ¶[0051] and Fig. 1).

Frondoza uses the two-component multi-well culture plate system to separate the insert component from the microtiter plate component to get drainage of the fluid of the culture medium, separating the tissue analogs in the insert from the medium in the microtiter plate. All wells are drained together. Frondoza states that his two-component system gives separation of the tissue analogs from the medium in each well “...without requiring a centrifugation or sedimentation step.” Ibid., ¶[0052] and ¶[0088]; emphasis added.

Frondoza uses the tissue for in vitro test systems for screening drugs for acute toxicity, rather than using primary healthy tissue or animals (Ibid., ¶[0037]). The tissue analogs can be further treated to induce disease-like conditions, and the resulting disease tissue analogs are used to screen for potential therapeutic drugs that modify the physiology or block progression of the disease condition (Ibid., ¶[0037]).

Frondoza fails to teach or suggest an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit the liquid to flow through the receptacle, to which claims 1 and 12 are directed. In contrast to the subject matter of claims 1 and 12, Frondoza merely shows an insert component having at the bottom of each well a porous screen that covers the bottom of the well of the insert component (Ibid., paragraph [0051]). No part of the walls of Frondoza’s insert component are permeable, i.e., liquid does not flow through the walls of the insert component (Ibid., column 4 paragraph [0051] and Figure 1). In fact, the Office action admits that Frondoza does not teach or suggest an apparatus in which a portion of the side walls of the receptacle are permeable. See Office action p. 4.

Therefore, Frondoza alone does not render obvious the subject matter of claims 1 and 12. Claims 3-11, 13-16, and 18 depend directly or indirectly from claims 1 or 12 and incorporate the subject matter of claims 1 or 12 and contain additional subject matter, and therefore these claims also are not obvious in view of Frondoza.

Applicants show below that Robinson does not cure the defects of Frondoza, alone or in any combination.

Robinson et al. (U.S. patent number 6,890,740 issued May 10, 2005)

Robinson shows an apparatus having a separation barrier for excluding denser cell materials from less dense cell materials and is used after centrifuging of cells or cell materials, so

that selected materials can be withdrawn from the less dense cell materials without inclusion of the denser cell materials (Robinson, Abstract). Factual analysis below demonstrates that Robinson's apparatus, either as a single mesh or as a barrier plate, is to be used as an insert in a centrifuge container after centrifugation of biological material.

Robinson shows that cell materials or cells from which selected material is to be withdrawn are centrifuged (Ibid., Abstract). Once the denser cell materials are isolated in a layer by centrifugal force, an "invention screen or sieve [sic]" is submerged in the less dense cell material to a level above the layer of denser cell materials, to isolate the denser cell materials from the less dense cell materials, preventing mixing of the denser cell materials back into the less dense cell materials after the cells or the cells in medium are no longer being centrifuged (Ibid., Abstract).

Robinson states:

To achieve the foregoing and other objects, and in accordance with the purposes of the present invention, as embodied and broadly described herein, there has been invented a separation barrier having a screen, mesh or sieve for excluding denser biological materials from less dense biological materials after centrifuging of the biological materials. [Ibid., column 1 line 66 to column 2 line 4; emphases added].

Robinson also shows that his apparatus and centrifugation should be used to maintain separation of desired less dense biological material from more dense biological material, rather than using filtering, straining, or binding. Robin states:

It has been discovered that rather than filtering, straining, binding, or otherwise processing denser biological materials to separate them from less dense biological materials, a separation barrier having a screen, mesh or sieve projecting in a conformation that will fit into a container in which biological material is centrifuged can be used to isolate the layer of denser materials which results from centrifuging the biological material from the supernatant. [Ibid., column 2 lines 50-57; emphases added].

Robinson shows two types of apparatus. One type of apparatus is a meshed well or single mesh, such as a simple stiff net that can fit into a centrifuge container (Ibid., column 3 lines 9-21 and Fig. 1). This type of apparatus is permeable throughout (Ibid., Fig. 1). Further, Robinson shows another type of apparatus that is a barrier plate having a bottom that has an open

mesh (Ibid., column 3 lines 23-35 and Fig. 2). This type of apparatus shown in Robinson has walls that are not permeable, i.e., liquid does not flow through the walls of the barrier plate.

Robinson fails to teach or suggest an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit liquid to flow through the receptacle, to which claims 1 and 12 are directed. In contrast to the subject matter of claims 1 and 12, Robinson shows two configurations for his apparatus, either as a single mesh that is permeable throughout (Ibid., Fig. 1), or as a barrier plate in which the walls of the barrier plate are not permeable (Ibid., Fig. 2). Neither configuration alone, nor the combination of each configuration, teaches or suggests a receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side, to which claims 1 and 12 are directed. Therefore Robinson fails to cure the defects of Frondoza.

For these reasons Frondoza and Robinson, alone or in any combination, do not render obvious the subject matter of claims 1 and 12. Claims 3-11, 13-16, and 18 depend directly or indirectly from claims 1 or 12 and incorporate the subject matter of claims 1 or 12 and contain additional subject matter, and therefore these claims also are not obvious in view of the combination of Frondoza and Robinson.

We further show below that the combination of these references would not have been motivating to one of ordinary skill in the art at the time the present application was filed to make the invention of the claims as presently presented.

#### Legal analysis of references combined

According to a summary of criteria in the *Manual of Patent Examining Procedure*, "[t]o establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure." [emphases

added] *Manual of Patent Examining Procedure* §2142 (8th Ed. Rev.2, May 2, 2004, “hereinafter M.P.E.P.”); *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

A recent decision by the U.S. Supreme Court, *KSR International Co. v. Teleflex Inc.* 550 U.S. \_\_\_\_ (2007), discusses criteria for showing a motivation to combine numerous prior art references in a determination that a claimed invention is obvious. The U.S. Supreme Court in *KSR* explained that “[t]here is no necessary inconsistency between the idea underlying the TSM [teaching, success, motivation] test and the *Graham* analysis.” *KSR International Co.* 550 U.S. \_\_\_\_ at p. 15. In fact, the court explains “... it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the newly claimed invention does.” Id.

Applicants respectfully traverse the above rejection, and show that the facts of the case and the relevant case law indicate that the invention would not have been obvious to one of ordinary skill in the art at the time the application was filed because the underlying facts show that the criteria for a *prima facie* rejection have not been met.

#### Failure of the cited prior art to teach or suggest all the claim limitations

To establish a *prima facie* case for obviousness of a claimed invention, all of the claim limitations must be taught or suggested by the prior art. M.P.E.P. §2143.03; *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974).

Claims 1 and 12 are directed *inter alia* to an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit liquid to flow through the receptacle. As shown below, none of the cited references teach or suggest this subject matter of claim 1 and claim 12.

Frondoza as characterized above shows a two-component multi-well culture plate system for culturing of cells to form tissue analogs (*Ibid.*, ¶[0051] and Fig. 1). One component is an insert having molded wells and an open bottom (*Ibid.*, ¶[0051] and Fig. 1). The open bottom of each well is fitted with a porous screen that covers the bottom of each well (*Ibid.*, ¶[0051] and Fig. 1). The other component is a standard microtiter plate (*Ibid.*, ¶[0051] and Fig. 1), and the wells of the insert fit into the wells of the microtiter plate to form the two-component multi-well culture plate system (*Ibid.*, ¶[0051] and Fig. 1). The walls of Frondoza’s insert component are

not permeable, i.e., liquid does not flow through the walls of the insert component (*Ibid.*, column 4 paragraph [0051] and Figure 1).

Robinson as characterized above shows two types of apparatus. One type of apparatus Robinson shows is a single mesh, such as a stiff net that can fit into a centrifuge container (*Ibid.*, column 3 lines 9-21 and Fig. 1). This type of apparatus shown in Robinson is permeable throughout (*Ibid.*, Fig. 1). Alternatively, Robinson shows another type of apparatus that is a barrier plate having a bottom that has an open mesh (*Ibid.*, column 3 lines 23-35 and Fig. 2). This type of apparatus shown in Robinson has walls that are not permeable, i.e., liquid does not flow through the walls of the barrier plate.

Factual analyses above demonstrate that one of ordinary skill in the art, combining the apparatus in Frondoza and the apparatus in Robinson, would have arrived at an apparatus that is merely either permeable throughout or is permeable only on the bottom. One of ordinary skill in the art, combining the apparatus in Frondoza with the apparatuses in Robinson, would not have been taught or given the suggestion of a receptacle that is permeable on the bottom and on a portion of at least one side, to which claims 1 and 12 are directed.

To suggest that a combination of an apparatus that is permeable throughout with an apparatus that is permeable only on the bottom would teach or suggest an apparatus that is permeable on the bottom and on a portion of at least one side is using Applicants' own specification as a blueprint to attempt to reconstruct the invention, which is impermissible hindsight.

Therefore, by the legal criteria discussed above, the underlying facts of the content of the cited prior art and of the present pending claims show that the prior art fails to teach or suggest all the limitations of the claims of the present invention. Therefore, a *prima facie* case that claims 1 and 12 of the present invention are obvious has not been made.

Claims 3-11, 13-16, and 18 that depend directly or indirectly from claims 1 or 12 and incorporate all of the subject matter of claims 1 or 12 and contain additional subject matter also are not obvious in light of the cited references.

For at least this reason, obviousness of the claims has not been established.

#### Lack of motivation to combine the cited prior art

To establish a *prima facie* case of obviousness, a reasonable expectation of success must be found in the prior art, and not based on Applicants' disclosure. The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills*, 916 F.2d 680, 16 USPDQ 1430 (Fed. Cir. 1990). The U.S. Supreme Court in *KSR International Co. v. Teleflex Inc.* 550 U.S. \_\_\_\_ (2007), a decision that issued on April 30, 2007, affirmed the legal principle that the mere fact that each element of a claimed invention could be found within the prior art does not render the claimed invention obvious. The court stated:

.... A patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. *KSR International Co.* 550 U.S. \_\_\_\_ at p. 14

"A prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention." M.P.E.P. §2141.02 citing *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540 (Fed. Cir. 1983) [emphasis in original]. This legal principle was affirmed by the U.S. Supreme Court in *KSR* when the court stated, "...when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious." *KSR International Co.* 550 U.S. \_\_\_\_ at p. 12, [emphases added]. Applicants show below that Robinson teaches away from combining his apparatuses with Frondoza's apparatus.

Robinson's apparatus is used after centrifuging of cells or cellular material, and is a separation barrier for excluding denser cell materials from less dense cell materials after centrifuging of the cells (Robinson, Abstract). Robinson shows that when the biological material is centrifuged, a layer of denser materials (pellet) such as broken cell walls, cell membranes, mitochondria and ribosomes forms at the outermost position of the centrifuge container, and an upper portion (supernatant) of less dense material has water, enzymes, soluble proteins and small DNA fragments (*Ibid.*, column 2 line 64 to column 3 line 3).

Robinson states:

To achieve the foregoing and other objects, and in accordance with the purposes of the present invention, as embodied and broadly described herein, there has been invented a separation barrier having a screen, mesh or sieve for excluding denser biological materials from less dense biological materials after centrifuging

of the biological materials. [Ibid., column 1 line 66 to column 2 line 4; emphases added].

Robinson clearly states:

It has been discovered that rather than filtering, straining, binding, or otherwise processing denser biological materials to separate them from less dense biological materials, a separation barrier having a screen, mesh or sieve projecting in a conformation that will fit into a container in which biological material is centrifuged can be used to isolate the layer of denser materials which results from centrifuging the biological material from the supernatant. [Ibid., column 2 lines 50-57; emphases added].

Thus Robinson's apparatus, either as a single mesh or as a barrier plate, is used after centrifugation. Robinson clearly teaches away from filtering, straining, and binding.

In contrast to Robinson, Frondoza's two-component multi-well culture plate system for cell culturing results in drainage of the fluid components of the culture medium into the microtiter well, separating the tissue analogs in the insert from the medium in the microtiter plate. Frondoza clearly states that his two-component system separates the tissue analogs in the insert from the medium in the microtiter plate "...without requiring a centrifugation or sedimentation step." Ibid., ¶[0052] and ¶[0088]; emphasis added.

Frondoza's apparatus is used without centrifugation because Frondoza needs intact tissue analogs, viz, healthy unbroken masses of cells, for screening drugs, for testing toxicity of chemical agents, or for genomic or proteomic screening (Ibid., Abstract). Centrifuging the cells might adversely affect cell viability or tissue analog integrity.

One of ordinary skill in the art, reading Robinson, would not have been motivated to combine Robinson's apparatus with Frondoza's apparatus because Robinson's apparatus is designed to be used after centrifugation, and Frondoza's apparatus is designed to be used without centrifugation.

Most important, Robinson clearly states that his apparatus is used with centrifugation, rather than filtering, straining, or binding (Robinson, column 2 lines 50-57).

In contrast, Frondoza clearly states that separation of the insert from the microtiter plate is for drainage of the fluid components of the culture medium into the microtiter well,

“...without requiring a centrifugation or sedimentation step.” Ibid., ¶[0052] and ¶[0088]; emphasis added.

From these statements in each of Robinson and Frondoza, the ordinarily skilled user simply would not have been motivated to combine Robinson with Frondoza. In fact, one of ordinary skill in the art would have been taught away from combining Robinson’s apparatus with Frondoza’s apparatus, because Robinson’s apparatus is used after centrifugation and Frondoza does not centrifuge. In fact, Frondoza is draining or straining.

By the legal criteria discussed above, the underlying facts of the content of the cited prior art show that Robinson teaches away from the combination with Frondoza, and Frondoza teaches away from the combination with Robinson. The prior art fails to teach or suggest to the ordinarily skilled artisan the desirability of the combination, and further fails to teach or suggest to the ordinarily skilled artisan a reasonable expectation of success in making the combination of Robinson and Frondoza. Therefore, a *prima facie* case that claims 1 and 12 of the present invention are obvious has not been made.

Claims 3-11, 13-16, and 18 that depend directly or indirectly from claims 1 or 12 and incorporate all of the subject matter of claim 1 or claim 12 and contain additional subject matter also are not obvious in light of the cited references.

Applicants respectfully request withdrawal of rejection of claims 1, 3-16, and 18 under 35 U.S.C. §103(a) in view of the combination of Robinson and Frondoza.

#### Robinson and Feygin et al. in combination

The Office action on p. 5 ¶3 rejects claims 1, 3-7, 10-16, and 18 under 35 U.S.C. §103(a) in view of Feygin et al. (U.S. patent number 6,315,957, issued November 13, 2001) in combination with Robinson. Applicants respectfully traverse.

Robinson is characterized above. Feygin et al. fails to cure any of the defects of Robinson, alone or in any combination, as is shown below.

#### Feygin et al. (U.S. patent number 6,315,957, issued November 13, 2001)

Feygin shows a method and an article for segregating solid support media or other solids from liquid, while at the same time maintaining such solids and liquid in intimate contact as may be required for various processing or chemical operations (Feygin, Abstract). Feygin uses this

apparatus for carrying-out solid-phase synthesis of chemical compounds (Ibid., column 5 line 54 to column 6 line 19). Chemical operations link a reactive functionality to a solid support so the functionality can be modified and then cleaved (Ibid., Abstract and Fig. 7). The liquid in Feygin includes chemical building blocks for reacting with the reactive functionality, so that the reaction produces a chemical compound (Ibid., column 2 lines 16-27).

Feygin's apparatus for segregating solid support media from liquid has a filter pocket plate consisting of a plate with a plurality of holes in which a mesh-like material projects through each hole in the plate (Ibid., column 1 lines 63-67, and Fig. 2). The pocket plate engages a vessel having wells suitable for retaining liquid (Ibid., column 2 line 4-10, and Fig. 3). Feygin's filter pocket plate is permeable throughout (Ibid., Fig. 2 and Fig. 3). Feygin's filter pocket plate is filtering.

Nowhere does Feygin teach or suggest an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit the liquid to flow through the receptacle, which is the subject matter of claims 1 and 12. In contrast to the subject matter of claims 1 and 12, Feygin's filter pocket plate is permeable throughout (Ibid., Fig. 2 and Fig. 3). In fact, the Office action admits that Feygin does not teach or suggest an apparatus in which a portion of the side walls of the receptacle are permeable. See Office action p. 5.

Therefore, Feygin does not cure the defects of Robinson, and the combination of Feygin and Robinson does not render obvious the subject matter of claims 1 and 12. Claims 3-7, 11, 13-16, and 18 depend directly or indirectly from claims 1 or 12 and incorporate the subject matter of claims 1 or 12 and contain additional subject matter, and therefore these claims also are not obvious in view of the combination of Feygin and Robinson.

As discussed above, a *prima facie* case of obviousness requires some suggestion or motivation to modify the reference or to combine reference teachings, a reasonable expectation of success, and that the prior art reference (or references when combined) must teach or suggest all the claim limitations.

Claims 1 and 12 are directed *inter alia* to an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit liquid to flow through the

receptacle. As shown below, none of the cited references teach or suggest this subject matter of claim 1 and claim 12.

Feygin's article for segregating solid support media from liquid has a filter pocket plate consisting of a plate with a plurality of holes in which a mesh-like material projects through each hole in the plate (Ibid., Abstract, column 1 lines 63-67, and Fig. 2). Feygin's filter pocket plate is permeable throughout (Ibid., Fig. 2 and Fig. 3), and Feygin is filtering.

Robinson shows two types of apparatus. One type of apparatus in Robinson is a single mesh insert for a centrifuge container (Ibid., column 3 lines 9-21 and Fig. 1). This apparatus is permeable throughout (Ibid., Fig. 1). Robinson also shows an apparatus that is a barrier plate having a bottom that has an open mesh (Ibid., column 3 lines 23-35 and Fig. 2). This apparatus has walls that are not permeable, i.e., liquid does not flow through the walls of the barrier plate.

Factual analysis above demonstrates one of ordinary skill in the art, combining the apparatus in Feygin and the apparatuses in Robinson, would have merely arrived at an apparatus that is either permeable throughout or an apparatus that is permeable only on the bottom. One of ordinary skill in the art, combining the apparatus in Feygin with the apparatuses in Robinson, would not have been taught or given the suggestion of a receptacle that is permeable on the bottom and on a portion of at least one side, to which claims 1 and 12 are directed.

To suggest that a combination of an apparatus that is permeable throughout with an apparatus that is permeable only on the bottom would teach or suggest an apparatus that is permeable on the bottom and on a portion of at least one side is using Applicants' own specification as a blueprint to attempt to reconstruct the invention, which is impermissible hindsight.

Therefore none of the cited prior art references teach or suggest this subject matter of claims 1 and 12 and a *prima facie* case of obviousness has not be established. Claims 3-7, 11, 13-16, and 18 depend directly or indirectly from claims 1 and 12 and include the subject matter of these claims and further include additional subject matter. Therefore Feygin in combination with Robinson also fails to render obvious claims 3-7, 11, 13-16, and 18.

Further, Applicants show below that neither Feygin nor Robinson, taken as a whole at the time the application was filed, would have motivated or suggested to one of ordinary skill, at the time the present application was filed, combining these references, let alone suggesting the

combination would have been successful, therefore not having motivated the invention of the claims.

Robinson states:

It has been discovered that rather than filtering, straining, binding, or otherwise processing denser biological materials to separate them from less dense biological materials, a separation barrier having a screen, mesh or sieve projecting in a conformation that will fit into a container in which biological material is centrifuged can be used to isolate the layer of denser materials which results from centrifuging the biological material from the supernatant. [Robinson, column 2 lines 50-57; emphases added].

Thus Robinson's apparatus is used with centrifugation, rather than filtering, straining, or binding.

In contrast, Feygin et al. shows an article for segregating solid support media from liquid (Feygin et al. Abstract). The article includes a filter pocket plate consisting of a plate with a plurality of holes in which a mesh-like material projects through each hole in the plate (Ibid., column 1 lines 63-67, and Fig. 2). Feygin is filtering.

Because Robinson uses his apparatus with centrifugation to maintain separation of materials already centrifuged, rather than using filtering, straining, or binding, and Feygin's apparatus is a filter pocket plate to cause separation, the ordinarily skilled user simply would not have been motivated to combine Robinson with Feygin. In fact, one of ordinary skill in the art would have been taught away from combining Robinson's apparatus with Feygin's apparatus because Robinson teaches away from filtering to maintain separation of materials and Feygin shows a filter pocket plate to filter and thereby cause separation.

Therefore, by the legal criteria discussed above, the underlying facts of the content of the cited prior art show that Robinson teaches away from the apparatus in Feygin. Therefore the prior art fails to teach or suggest to the ordinarily skilled artisan the desirability of the combination, and further fails to teach or suggest to the ordinarily skilled artisan a reasonable expectation of success in making the combination of Robinson and Feygin. Therefore, a *prima facie* case that claims 1 and 12 of the present invention are obvious has not been made.

Claims 3-7, 11, 13-16, and 18 that depend directly or indirectly from claims 1 or 12 and incorporate all of the subject matter of claim 1 or claim 12 and contain additional subject matter also are not obvious in light of the cited references.

Applicants respectfully request withdrawal of rejection of claims 1, 3-7, 11-16, and 18 under 35 U.S.C. §103(a) in view of the combination of Robinson and Feygin.

Robinson, Feygin et al., Reeve et al., and/or Valkirs et al. in combination

The Office Action on pp. 6-7 ¶5 rejects claims 1, 3-7, 10-16, and 18-20 under 35 U.S.C. §103(a) in view of Feygin et al. in combination with Robinson, Reeve et al. (International patent application number WO 91/12079, published August 22, 1991) and/or Valkirs et al. (U.S. patent number 6,348,318, issued February 19, 2002). Applicants respectfully traverse.

Robinson and Feygin are characterized above. Reeve and Valkirs et al. fail to cure any of the defects of Robinson and Feygin, alone or in any combination, as is shown below.

Reeve et al. (Internation patent application number WO 91/12079, published August 22, 1991)

Reeve et al. shows methods of isolating macromolecules using magnetically attractable beads. In fact, Reeve et al. is titled, “[m]ethod to isolate macromolecules using magnetically attractable beads which do not specifically bind the macromolecules.” [emphasis added].

Reeve shows a method using magnetically attractable beads, the method includes the steps of: suspending magnetically attractable beads in a solution of a polymer; and applying a magnetic field to draw down a precipitate of the beads and the associated polymer (Reeve, p. 4 lines 6-20). In fact, Reeve states, “[t]he key to the invention is the use of magnetically attractable beads ...” (Ibid., p. 4 lines 21-23; emphases added). Reeve shows only separation methods based on use of magnetic beads and application of a magnetic field (Ibid., p. 6-16).

The separation method used in Reeve is based on an entirely different technology using different physical principles than the apparatus of the present claims or any of the other cited references above. Factual analysis shows that the separation method used in Reeve requires magnetically attractable beads and application of a magnetic field.

In contrast, in the apparatus of the present claims for intermixing objects and a liquid, retains the objects in the receptacle and the liquid flows through permeable portions of the receptacle, which is completely different than what is shown in Reeve.

Reeve does not teach or suggest any receptacle that is permeable of any type. Reeve does not teach or suggest a vessel into which is inserted the receptacle that is permeable.

Most important, Reeve fails to teach or suggest an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit said liquid to flow through the receptacle, with the proviso that a portion of at least one side is not permeable, which is the subject matter of claims 1 and 12.

Therefore, Reeve does not cure the defects of Feygin and Robinson, alone or in combination, and fails to render obvious the subject matter of claims 1 and 12. Applicants show below that Valkirs, alone or in combination, does not cure the defects of Feygin, Robinson, and Reeve in combination.

Valkirs et al. (U.S. patent number 6,348,318, issued February 19, 2002)

Valkirs shows a method that uses magnetic particles to concentrate target analytes (Valkirs, Abstract, and Fig. 1). Valkirs is titled, “[m]ethods for concentrating ligands using magnetic particles”. (emphasis added) Valkirs shows types of magnetic beads, for example, iron oxide particles, and commercial suppliers of magnetic beads (Ibid., column 5 lines 14-48).

Valkirs' method involves adding a magnetic bead having attached a capture moiety to a sample that has a target analyte binding moiety to form a magnetic bead-bound target complex (Ibid., column 1 lines 54-61). A magnetic field is applied to the sample to collect the magnetic bead-bound target complex (Ibid., column 1 lines 61-66). Valkirs' beads are binding the target.

Separation of Valkirs' target molecules requires a magnetic field to concentrate the sample at the bottom of a container. The liquid is then aspirated or poured from the container (Ibid., column 12 lines 1-14).

The separation method used in Valkirs is based on an entirely different technology using different physical principles than the apparatus of the present claims or any of the references cited above. Factual analysis shows that the separation method used in Valkirs requires magnetically attractable beads and application of a magnetic field.

In contrast, the apparatus of the present claims for intermixing objects and a liquid retains objects in the receptacle and the liquid flows through permeable portions of the receptacle, which is a completely different apparatus than what is shown in Valkirs.

Valkirs does not teach or suggest any receptacle that is permeable of any type. Valkirs does not teach or suggest a vessel to which is inserted the receptacle that is permeable.

Most important, Valkirs fails to teach or suggest an apparatus for intermixing objects and a liquid including at least one receptacle having sides and a bottom in which the receptacle is permeable on the bottom and on a portion of at least one side to permit said liquid to flow through the receptacle, with the proviso that a portion of at least one side is not permeable, which is the subject matter of claims 1 and 12.

Therefore, Valkirs does not cure the defects of Feygin, Robinson, and Reeve, alone or in combination, and fails to render obvious the subject matter of claims 1 and 12. Claims 3-7, 10-11, 13-16, and 18-20 depend directly or indirectly from claims 1 or 12 and incorporate the subject matter of claims 1 or 12 as here amended and contain additional subject matter, and therefore these claims also are not obvious in view of the combination of Feygin, Robinson, Reeve, and Valkirs.

As discussed above, a *prima facie* case of obviousness requires some suggestion or motivation to modify the reference or to combine reference teachings, a reasonable expectation of success, and that the prior art reference (or references when combined) must teach or suggest all the claim limitations.

Factual analysis above demonstrates that one of ordinary skill in the art, combining the apparatus in Feygin and Robinson, would have merely arrived at an apparatus that is either permeable throughout or an apparatus that is permeable only on the bottom. One of ordinary skill in the art, combining the apparatus in Feygin with the apparatuses in Robinson, would not have been taught or given the suggestion of a receptacle that is permeable on the bottom and on a portion of at least one side, to which claims 1 and 12 are directed.

Reeve and Valkirs fail to cure this defect of the combination of Feygin and Robinson because Reeve and Valkirs teach and suggest only a vessel that is non-permeable. In fact, nothing in Reeve and Valkirs teaches or suggests any receptacle that is permeable of any type, because using a magnetic system eliminates need for a vessel.

Therefore the combination of Feygin, Robinson, Reeve, and Valkirs fails to teach or suggest all the claim limitations as required by M.P.E.P. §2142. Therefore a *prima facie* case of obviousness of claims 1 and 12 has not been made. Claims 3-7, 10-11, 13-16, and 18-20 that depend directly or indirectly from claims 1 or 12 and incorporate all of the subject matter of

claims 1 or 12 and contain additional subject matter also are not obvious in light of the cited references.

Further, Applicants show below that none of the cited references, taken as a whole at the time the application was filed, would have motivated or suggested to one of ordinary skill, at the time the present application was filed, combining these references, let alone suggest that the combination would have been successful, therefore not motivating the invention of the claims.

Robinson states:

It has been discovered that rather than filtering, straining, binding, or otherwise processing denser biological materials to separate them from less dense biological materials, a separation barrier having a screen, mesh or sieve projecting in a conformation that will fit into a container in which biological material is centrifuged can be used to isolate the layer of denser materials which results from centrifuging the biological material from the supernatant. [Robinson, column 2 lines 50-57; emphases added].

Robinson's apparatus is used with centrifugation, rather than filtering, straining, or binding, to maintain separation.

In contrast to Robinson, Feygin et al. shows an article for segregating solid support media from liquid (Feygin et al. Abstract). The article includes a filter pocket plate consisting of a plate with a plurality of holes in which a mesh-like material projects through each hole in the plate (Ibid., column 1 lines 63-67, and Fig. 2). Feygin is filtering.

Reeve shows a method of treating a solution of a polymer with magnetically attractable beads, to draw down a precipitate of the beads and the associated polymer (Reeve, p. 4 lines 6-20). Reeve states, “[t]he key to the invention is the use of magnetically attractable beads ...” (Ibid., p. 4 lines 21-23; emphases added). Reeve is binding

Valkirs shows a method that uses magnetic particles to concentrate target analytes (Valkirs, Abstract, and Fig. 1). The Valkirs' method involves adding a magnetic bead having attached a capture moiety to a sample that has a target analyte binding moiety to form a magnetic bead-bound target complex (Ibid., column 1 lines 54-61). A magnetic field is applied to the sample to collect the magnetic bead-bound target complex (Ibid., column 1 lines 61-66). Valkirs is binding.

There would have been no motivation to combine Robinson with Feygin, Reeve, and Valkirs because Robinson states that his apparatus is used after centrifugation and centrifugation is used to separate denser biological material from less dense biological material, rather than using filtering, straining, or binding. The apparatus maintains the separation. Feygin shows a filter pocket plate to cause separation by filtering, and each of Reeve and Valkirs show separation by binding to magnetic particles. In fact, one of ordinary skill in the art would have been taught away from combining Robinson's apparatuses with the apparatuses of any of Feygin, Reeve and Valkirs in any combination because Robinson shows, teaches and suggests not using filtering, straining, or binding, while Feygin, Reeve, and Valkirs show a filter pocket plate and binding to magnetic particles, respectively.

Therefore, by the legal criteria discussed above, the underlying facts of the content of the cited prior art show that Robinson teaches away from the combination of the apparatus in Robinson with the apparatus in each of Feygin, Reeve, and Valkirs. Therefore the prior art failed to teach or suggest to the ordinarily skilled artisan the desirability of the combination, and further failed to teach or suggest to the ordinarily skilled artisan a reasonable expectation of success in making the combination of Robinson, Feygin, Reeve, and Valkirs. Therefore, a *prima facie* case that claims 1 and 12 of the present invention are obvious has not been made.

Claims 3-7, 10-11, 13-16, and 18-20 that depend directly or indirectly from claims 1 or 12 and incorporate all of the subject matter of claim 1 or claim 12 and contain additional subject matter also are not obvious in light of the cited references.

Applicants respectfully request withdrawal of rejection of claims 1, 3-7, 10-16, and 18-20 under 35 U.S.C. §103(a) in view of the combination of Robinson, Feygin, Reeve, and/or Valkirs.

Claims comply with 35 U.S.C. §112 ¶2

The Office action on p. 2 ¶2 rejects claims 1, 8, 9, and 12 under 35 U.S.C. §112 ¶2, alleging that the term "small" in claims 1 and 12 as it relates to the size and make up of the objects is indefinite.

As a preliminary matter, claims 1 and 12 were amended in the Response submitted June 8, 2007, removing the term "small" from these claims. See Response Submitted June 8, 2007 pp. 3-7, and 15-17.

Claim 1 in the Response submitted June 8, 2007 was amended as follows:

(currently amended) Apparatus for intermixing ~~small~~ objects and a liquid comprising at least one receptacle having sides and a bottom for receiving and retaining said ~~small~~ objects, wherein said ~~small~~ objects are at least one object selected from the group of affinity beads and one or more living organisms, at least a portion of wherein said receptacle ~~being~~ is permeable on the bottom and on a portion of at least one side to permit said liquid to flow through said portion receptacle to intermix with said affinity beads objects when said receptacle is inserted into a vessel containing said liquid, ~~wherein said liquid is a lysate containing proteins to be purified with the proviso that a portion of at least one side is not permeable.~~ [Underlining and strike-through present in Response submitted June 8, 2007]

Claim 12 in the Response submitted June 8, 2007 was amended as follows:

(currently amended) Apparatus for intermixing ~~small~~ objects with a liquid comprising a plurality of receptacles, each receptacle having sides and a bottom for containing said ~~small~~ objects, wherein said ~~small~~ objects are at least one object selected from the group of affinity beads and one or more living organisms, each of said receptacles is permeable on the bottom and on a portion of at least one side having a lower portion thereof that is permeable to permit the flow of said liquid therethrough, with the proviso that a portion of at least one side is not permeable, wherein ~~said liquid is a lysate containing a protein to be purified, a vessel containing said solution, and means for repeatedly inserting said receptacles into said liquid in said vessel and then withdrawing said receptacle from said liquid to cause said liquid to first flow into said receptacle through said lower permeable portion when said receptacle is inserted into said liquid and then to flow outwardly from said receptacle through said lower permeable portion when said receptacle is withdrawn from said liquid.~~ [Underlining and strike-through present in Response submitted June 8, 2007]

Thus, claims 1 and 12 as here presented no longer include the term "small". Applicants respectfully request further explanation.

Further, Applicants show below that claims 1 and 12 as here presented comply with the requirements of 35 U.S.C. §112 ¶2.

One of ordinary skill in the art of biochemistry, reading the specification at the time it was filed, would have understood the size of the objects in claims 1 and 12 as presented, as shown below. The specification as filed on p. 7 paragraph [024] shows an example of a type of affinity bead, viz., TALON beads (commercially available from Biosciences Clontech). One of ordinary skill in the art of biochemistry would have understood the size of these commercially

available affinity beads at the time the application was filed because the commercial literature is replete with this information. See Appendix A attached hereto which is an excerpt from the BD Biosciences Clontech catalog describing TALON affinity beads. This catalog shows exemplary affinity bead size ranges, such as, 45 $\mu$ m to 165 $\mu$ m, 60 $\mu$ m to 160 $\mu$ m, and 300 $\mu$ m to 500 $\mu$ m. See Appendix A, p. 13.

Further, the specification as filed on p. 8 paragraph [035] provides examples of living organisms, embryos and larvae. One of ordinary skill in the art of biochemistry would have been well informed regarding sizes of living organisms of choice, at the time the application was filed because the scientific literature is replete with this information. See Appendix B attached hereto, Dong et al. (Crop Science 43(3): 1068-1071, 2003). This article shows exemplary sizes of embryos, viz., widths that range from about 300 $\mu$ m to about 560 $\mu$ m and lengths that range from about 1300 $\mu$ m to about 1900 $\mu$ m. See Appendix B, enlarged view of Fig. 1 on last page of article.

In fact, the specification as filed on p. 5 paragraphs [018] to [019] shows exemplary pore sizes for the permeable portion of the receptacle, for example, 25 $\mu$ m, 30 $\mu$ m, 33 $\mu$ m, and 41 $\mu$ m. One of ordinary skill in the art of biochemistry would have understood, reading the application at the time it was filed, to choose a pore size that is smaller than the size of the objects so that the receptacle is capable of receiving and retaining the objects of claims 1 and 12 as presented.

For these reasons, one of ordinary skill in the art in biology or biochemistry, reading the specification at the time the application was filed, would clearly have understood the size of the objects that are the subject matter of claims 1 and 12 as presented.

Applicants respectfully request withdrawal of rejection of claims 1 and 12 under 35 U.S.C. §112 ¶2.

The Office action on p. 2 ¶2 rejects claims 1, 8, and 9 under 35 U.S.C. §112 ¶2 alleging claims 8 and 9 lack antecedent basis as they depend from claim 1 because claim 1 defines “small objects” as affinity beads and claims 8 and 9 define the “small objects” as living organisms.

As a preliminary matter, claims 1, 8, and 9 were amended in the Response submitted June 8, 2007, to remove the term “small” from each of these claims. See Response Submitted June 8, 2007 pp. 3-7, and 15-17. Claim 1 was further amended to include the term “living organisms”. See Response Submitted June 8, 2007 pp. 3-7, and 15-17.

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Amendment and Response

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Date of Deposit: October 22, 2007

Claim 1 as amended in the Response submitted June 8, 2007 is provided above. Claim 8 in the Response submitted June 8, 2007 was amended as follows:

(currently amended) Apparatus for intermixing ~~small~~ objects and a liquid as set forth in claim 1 wherein said ~~small~~ objects comprise one or more living organisms. [Underlining and strike-through present in Response submitted June 8, 2007]

Claim 9 in the Response submitted June 8, 2007 was amended as follows:

(currently amended) Apparatus for intermixing ~~small~~ objects and a liquid as set forth in claim 8 wherein said liquid contains a stain or a pharmaceutical, or said living organisms are subjected to biochemical conditions, whose effect on said living organism is being studied. [Underlining and strike-through present in Response submitted June 8, 2007]

Thus, claim 1 as previously presented provides proper antecedent basis for use of the term "living organism" in each of claims 8 and 9. Therefore, Applicants respectfully request withdrawal of rejection of claims 1, 8, and 9 under 35 U.S.C. §112 ¶2.

### Summary

On the basis of the foregoing reasons, Applicants respectfully submit that the pending claims are in condition for allowance, which is respectfully requested. If there are any questions regarding these remarks, the Examiners are invited and encouraged to contact Applicants' representatives at the telephone number provided.

Respectfully submitted,



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Dated: October 22, 2007

## **Appendix A**

# TALON™ Products

For polyhistidine-tagged protein purification

Product	Application
<b>TALON™ Metal Affinity Resin</b> Resin ready for loading in columns for small or medium-scale purification of His-tagged proteins. Purify > 5 mg protein using 1 ml of resin.	For purification of most cytosolic and secreted His-tagged proteins by small-scale or batch/gravity flow, under native or denaturing conditions.
<b>TALON™ Superflow Resin</b> Specially designed for quick and effective purification of His-tagged proteins at high flowrates and medium-pressure (up to 150 psi).	For FPLC, medium-pressure chromatography, or scale-up for production applications.
<b>TALON™ CellThru</b> Novel IMAC resin designed for quick purification of His-tagged proteins by direct capture.	For small-scale single-use applications such as verifying positive transformants for His-tagged protein expression levels, or trial-level purification protocols.
<b>TALONspin™ Columns</b> Ready-made spin columns containing TALON-NX™ resin for the simultaneous purification of several His-tagged proteins in parallel in only 30 minutes.	For small-scale single-use applications such as verifying positive transformants for His-tagged protein expression levels, or trial-level purification protocols.
<b>TALON™ Purification Kit</b> Convenient kit containing TALON resin, columns, and all the buffers necessary to extract, wash, and elute His-tagged proteins. This kit provides the ideal place to start when using TALON in your applications.	
<b>TALON™ Disposable Columns</b> Two different types of disposable columns—one for use with TALON Resin for regular His-tagged protein purification and one for use with CellThru Resin for purification from crude lysates.	
<b>TALON™ Buffer Kit</b> Supplemental kit containing concentrated forms of optimized buffers for extracting, washing, and eluting proteins.	

# TALON™ Metal Affinity Resin

TALON Resins are durable, cobalt-based IMAC resins designed to purify recombinant poly-histidine-tagged proteins (Bush *et al.*, 1991). These resins are compatible with many commonly used reagents, and allow protein purification under native and denaturing conditions. They can be used with all prokaryotic and eukaryotic expression systems in a variety of formats, including small-(mini-) scale batch screening, large-scale batch preparations, and methods using gravity-flow columns and spin columns.

## Introduction

Proteins have evolved very complex structures in order to perform a diverse array of functions. As a result, their physicochemical properties vary greatly, posing difficulties when developing versatile purification protocols. A host of purification methods have been developed that capitalize on the general physical properties of proteins. One of the quickest and easiest ways to purifying a protein is to use affinity chromatography since it is generally a more selective method of purification, which lets the protein of interest can be purified in one or two steps. However, many proteins have not been characterized sufficiently, or do not have any known strong binding properties that can be utilized for purification. One way to circumvent this problem is to incorporate a purification tag into the primary amino acid sequence of a target protein, thus constructing a recombinant protein with a binding site that allows purification under well-defined, generic conditions.

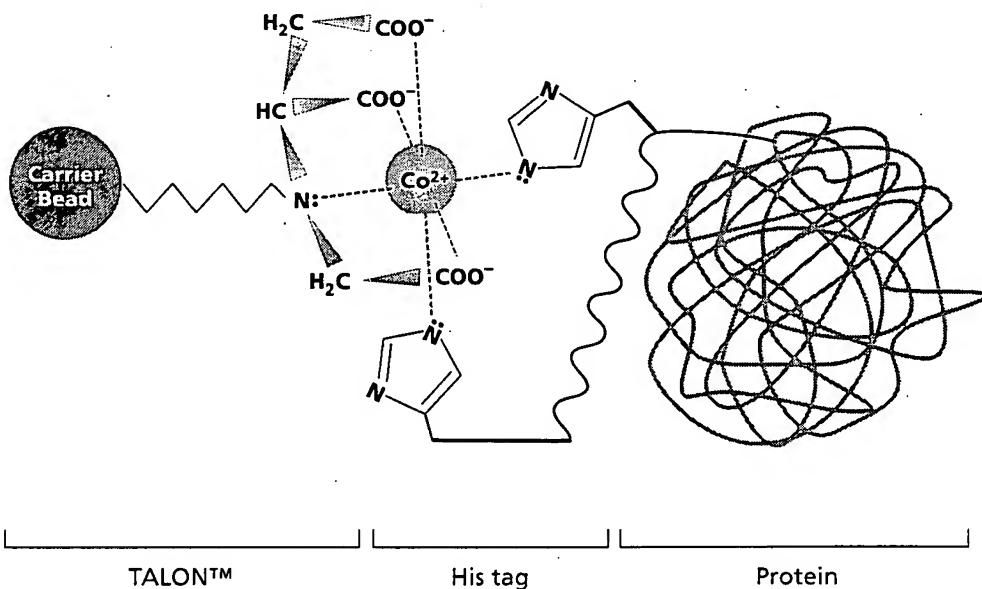


Figure 2. Molecular mechanism of histidine binding to TALON™ Resin.

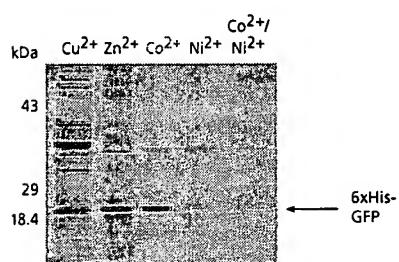
## TALON™ Metal Affinity Resin...cont.

### IMAC technology

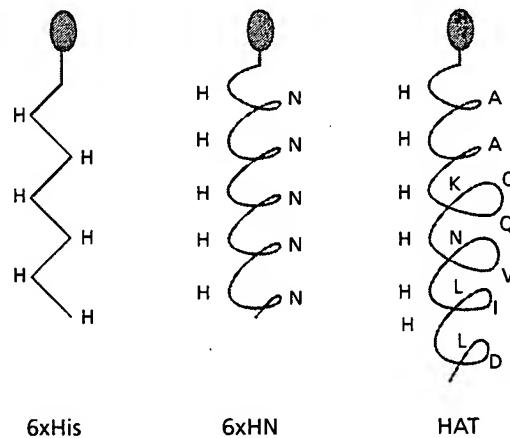
TALON Resin is an immobilized metal affinity chromatography (IMAC) resin based on our innovative, patented technology. IMAC was introduced in 1975 as a group-specific affinity technique for separating proteins (Porath *et al.*, 1975). This principle is based on the reversible interaction between various amino acid side chains and immobilized metal ions. Depending on the immobilized metal ion, different side chains can be involved in the adsorption process. Most notably, histidine, cysteine, and tryptophan side chains have been implicated in protein binding to immobilized transition metal ions and zinc (Porath, 1985; Sulkowski, 1985; Hemdan & Porath, 1985a; Hemdan & Porath, 1985b; Zhao *et al.*, 1991).

### His-tag purification

Histidines exhibit highly selective binding to certain metals and have great utility in IMAC. Under conditions of physiological pH, histidine binds by sharing electron density of the imidazole nitrogen with the electron-deficient orbitals of transition metals. Although only three histidines may bind transition metals under certain conditions, six histidines reliably bind transition metals in the presence of strong denaturants such as guanidinium (Hochuli *et al.*, 1987). Such protein tags are commonly referred to as 6xHis tags. We have developed several other His-tag purification systems, including 6xHN and HAT. These tags possess characteristics favorable for binding to IMAC resins and improve protein solubility and yield (see page 22).



**Figure 3.** The cobalt ion has higher affinity and specificity for His-tagged proteins. The indicated metals were immobilized onto sepharose CL-6B (Pharmacia) using TALON's unique tetradentate chelator. 20  $\mu$ l of eluate from the indicated resin was electrophoresed on a 12% polyacrylamide gel and stained with Coomassie blue.



**Figure 4.** Hypothetical structures of commonly used histidine-affinity tags.

# Unique Properties of TALON™ Resin

## Reactive core contains cobalt

TALON has a remarkable affinity and specificity for His-tagged proteins (Figures 3, 7, 9, 10, & 11). The TALON reactive core, which contains cobalt, has strict requirements for the spatial positioning of histidines. Only adjacent histidines or specially positioned, neighboring histidines are able to bind cobalt in this reactive core. In nickel-based resins (i.e. Ni-NTA Resin), these spatial requirements are less strict. Therefore, nickel-based resins are also able to bind histidines located in places other than the protein's His-tag (Figure 3).

## Uniform matrix

Cobalt-based resins have a more uniform structure than nickel-based resins. All reactive sites in TALON resin look like three-dimensional pockets, similar to the one drawn in Figure 2. In these pockets, cobalt is bound to three carboxyl groups and one nitrogen atom, and is able to bind to two other ligands, i.e. two histidines. In this configuration, cobalt is bound very tightly and does not leak out of the resin. Nickel-based resins are less homogeneous in structure because nickel ions can form two different coordination structures. One of them is a three-dimensional pocket, similar to TALON. The other structure is planar (flat). In this distorted, planar structure nickel is bound to only two carboxyl groups and one nitrogen atom. Since this binding is not very strong, planar reactive cores are not able to hold nickel ions very tightly. This leads to leaching of the nickel ion from the resin.

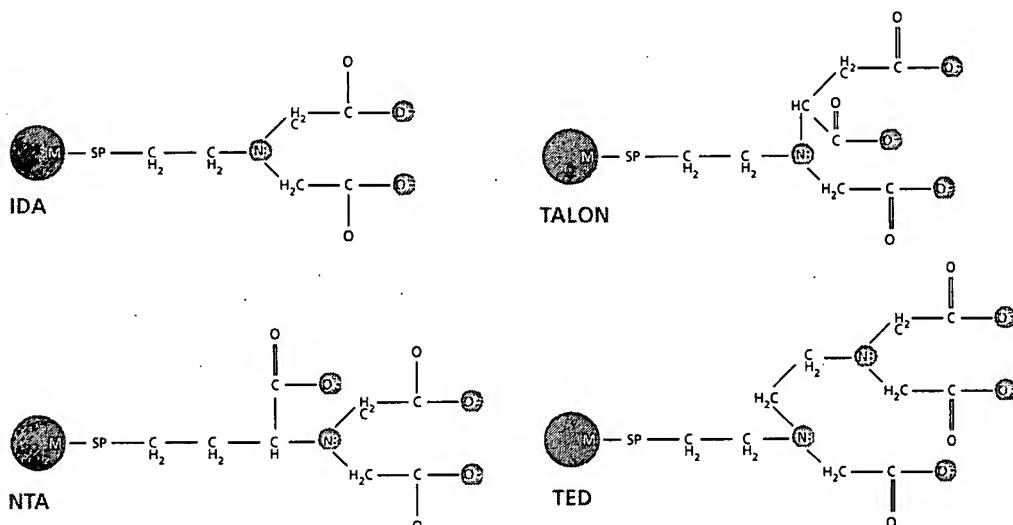


Figure 5. Chemical structures of chelating ligands used in IMAC. Binding groups are colored purple. SP = spacer. M = matrix.

## Unique Properties of TALON™ Resin...cont.

### Comparison with Ni-NTA resin

TALON exhibits subtle yet important differences in character when compared with nickel IMAC resins. For example, nickel-based IMAC resins often exhibit an undesirable tendency to bind unwanted host proteins containing exposed histidine residues (Kasher *et al.*, 1993). In contrast, TALON binds polyhistidine-tagged proteins with enhanced selectivity over nickel-based resins, and it also exhibits a significantly reduced affinity for host proteins (see page 6; Sulkowski, 1989). This characteristic offers two practical advantages. First, virtually no background proteins are bound to TALON when the sample is applied; consequently, cumbersome washing procedures are not generally required before protein elution. Second, polyhistidine-tagged proteins elute from TALON under slightly less stringent conditions—a slightly higher pH or lower imidazole concentration—than with nickel IMAC resins. Elution occurs when the imidazole nitrogen ( $pK_a$  of 5.97) is protonated, generating a positively charged ammonium ion, which is repelled by the positively charged metal atom. Alternatively, simply adding imidazole to the elution buffer can competitively elute the bound polyhistidine-tagged protein because imidazole is structurally identical to the histidine side chain and therefore out-competes histidines for resin binding.

#### Why metal leaching is detrimental to protein purification

During protein purification, metal separates from the reactive core of the purification resin and flows down the column. This is called metal leaching. When metal leaching occurs, it reduces the number of reactive sites available for protein binding on the column, therefore reducing the amount of purified protein obtained.

All metals will leach out of a resin, but nickel leaches more readily than cobalt. Nickel can also precipitate proteins by forming salt bridges, can be toxic to cells and tissues, and can damage purified protein because of its nucleophilic properties. For these reasons, TALON Resin employs cobalt in its reactive core rather than nickel.

Table I: Comparison of TALON™ Metal Affinity Resin vs. Ni-NTA Resin

	TALON™ Resin	Ni-NTA Resin
Metal	Cobalt	Nickel
Metal Ion Complex	Strong	Weak, metal leakage results in lower yields of His-tagged protein and contamination by nonspecific proteins
Sensitivity to $\beta$ -mercaptoethanol	Low to negligible sensitivity when concentration < 30 mM	High, resulting in low yields of His-tagged protein
Performance under denaturing conditions	++	+
Performance under nondenaturing conditions	+	+
Reusability	++	+
Nonspecific protein binding	None	Significant

# Protein Purification with TALON™

## Denaturing vs. native conditions

### Purification conditions

Deciding whether to use native or denaturing purification conditions depends on protein location, solubility, accessibility of the histidine tag, downstream applications, and preservation of biological activity. TALON Resin retains its protein binding specificity and yield in a variety of purification conditions. It is stable in both denaturing and native (nondenaturing) conditions.

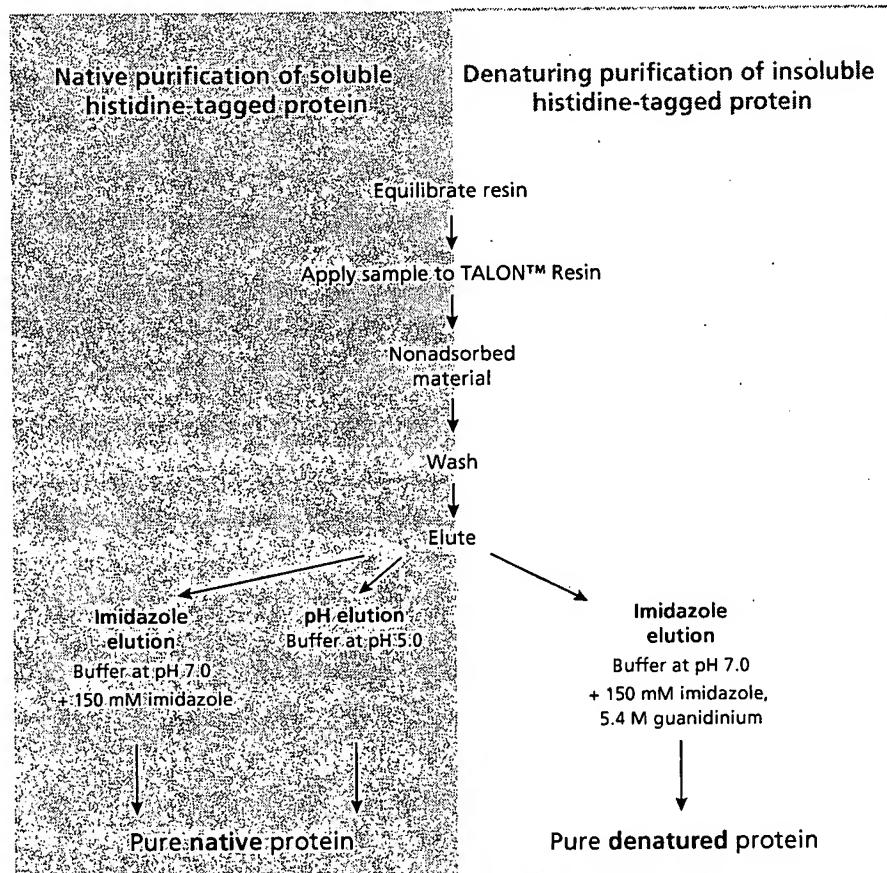


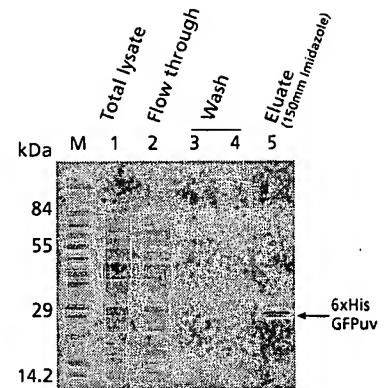
Figure 6. Native vs. denaturing purification procedures.

## Protein Purification with TALON™...cont.

### Why use denaturing conditions?

Denaturants, such as 6 M guanidinium, enhance protein solubility. Because proteins that are overexpressed in prokaryotic systems sometimes form insoluble aggregates called inclusion bodies, you may need to purify proteins under denaturing conditions. Strong denaturants such as 6 M guanidinium or 8 M urea completely solubilize inclusion bodies and 6xHis-tagged proteins. Under denaturing conditions, the 6xHis tag on a protein will be fully exposed so that binding to the matrix will improve, and the potential for nonspecific binding will be greatly reduced.

6xHis-tagged proteins purified under denaturing conditions can be used directly in subsequent applications, or may need to be renatured and refolded. Protein renaturation and refolding can be performed prior to elution from the column (Holzinger *et al.*, 1996) or in solution (Wingfield *et al.*, 1995). However, yields of recombinant proteins will be lower than under native conditions. This is because urea and guanidinium molecules compete with histidines for binding to metal.



**Figure 7. Purification of 6xHis-GFPuv under denaturing conditions.** The fusion protein was purified in 8-M urea using TALON resin. M=molecular weight markers.

### Protein Solubility

Protein solubility is largely dependent on two factors: the hydrophobicity of the amino acids in the polypeptide backbone, and the ability of the protein to fold correctly. Researchers can use a number of standard methods developed to influence protein solubility. At the level of protein expression, protein solubility can be changed by changing the level of expression. In *E. coli*, recombinant proteins that are overexpressed are frequently found to form protein aggregates called inclusion bodies. Such structures are believed to be masses of the expressed protein that have not folded correctly. Depending on your application, inclusion body formation can frequently be overcome by either reducing the level of expression. Alternatively, switching from 6xHis tag to HAT tag may help to increase protein solubility (see page 22 for details). Sometimes, switching to a eukaryotic expression system helps the solubility of expressed protein because eukaryotes have the ability to add post-translational modifications or utilize chaperonin-assisted protein folding. At the level of protein purification, solubility can be increased by changing the temperature or salt concentration, or using reducing agents and denaturants in the method.

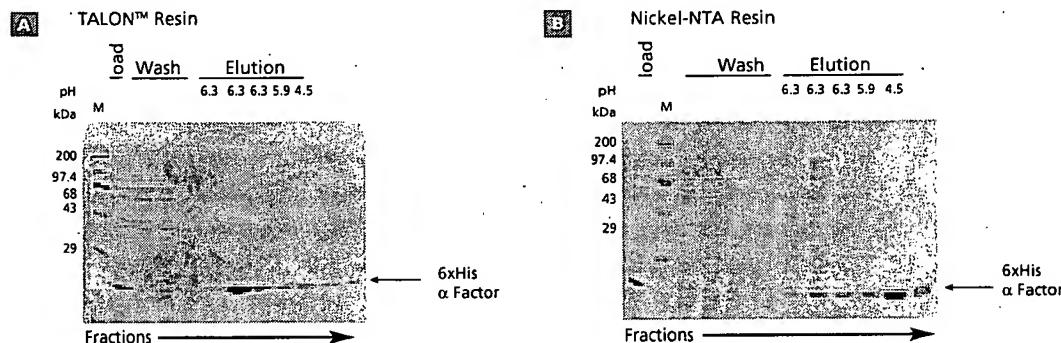
## Protein Purification with TALON™ ...cont.

### Why use native conditions?

Purifying a protein under native conditions is the most efficient method of retaining its biological activity. In order to use native conditions the protein must be soluble. Purification of proteins under native conditions is advantageous not only because you avoid the renaturation step at the end of the purification, but also because native purification will usually copurify enzyme subunits, cofactors, and associated proteins present in the cells (Le Grice, *et al.*, 1990; Flachmann & Khulbrandt, 1996). When renaturing protein after a denaturing purification, it is uncommon to regain more than 2–5% of the activity.

One disadvantage of using native conditions is that unrelated, nontagged proteins are more likely to be nonspecifically bound to the TALON Resin than with denaturing conditions. However, the nonspecific binding can be reduced by including a low concentration of imidazole (5–20 mM) in the wash buffer.

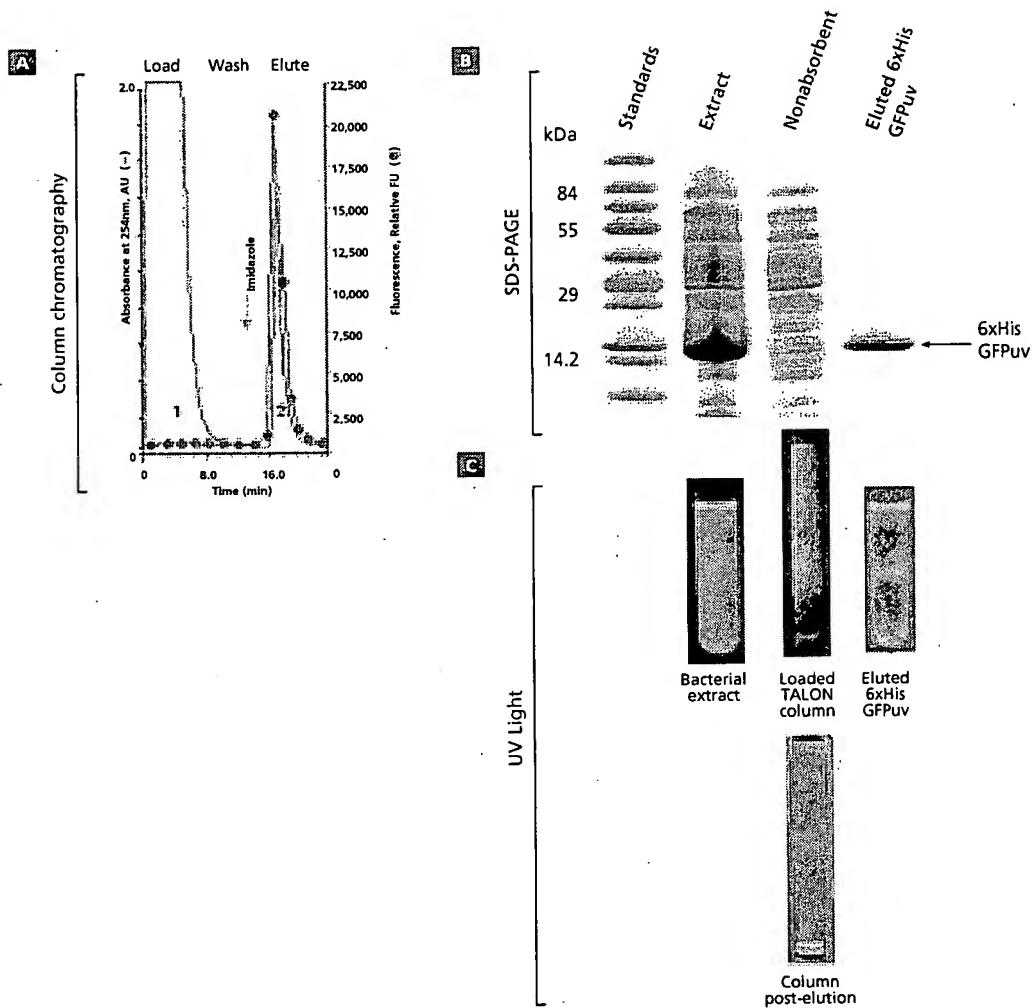
Sometimes the 6xHis tag is concealed by the tertiary structure of the soluble protein, so the protein must be denatured before it can be purified. If purification can only be performed under denaturing conditions, and this does not suit the downstream applications, an inaccessible tag can be moved to the other terminus of the protein. Alternatively, a larger tag like HAT or 6xHN can be used (see page 22).



**Figure 8. Purification of 6xHis proteins under native conditions compared to purification using Ni-NTA.** In comparison with Ni-NTA resin, TALON is more specific for His-tagged proteins. His-tagged proteins can be eluted from TALON at more neutral conditions (pH = 6.3) than from Ni-NTA resins (pH = 4.5). 6xHis-tagged prepro- $\alpha$ -factor was expressed in *E. coli*, lysed and loaded onto each gravity flow column and eluted by a step-wise pH gradient. Purified fractions were analyzed by SDS-PAGE. M=molecular weight markers.

## Protein Purification with TALON™...cont.

TALON resin preserves the native activity of purified proteins. Figure 9 shows that biological activity of green fluorescent protein (GFPuv) is preserved when purified using TALON Superflow Resin.

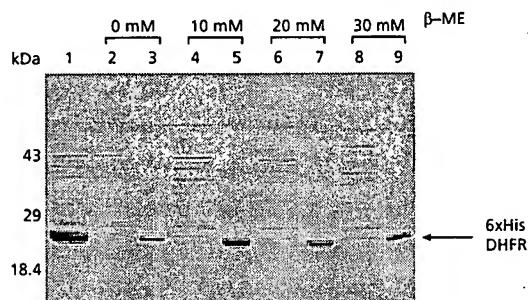


**Figure 9. Native purification with TALON™ preserves biological activity of proteins.** Fresh cells (0.5 g) expressing 6xHis-GFPuv were extracted in 5 ml of 50 mM sodium phosphate; 0.3 M NaCl, pH 7.0 Panel A. Elution profile of GFP which was loaded, washed with the same buffer, and eluted with a step gradient of imidazole (150 mM). Panel B. Fractions were analyzed by SDS-PAGE. Panel C. Active, intact GFP protein visualized under UV light.

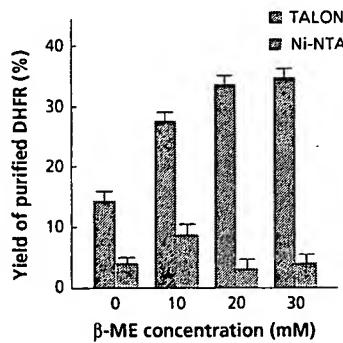
# Purification with $\beta$ -Mercaptoethanol

Why use  $\beta$ -mercaptopethanol in protein purification?

Some intracellular proteins contain reduced sulfhydryl (-SH) groups that are important for the biological activity and structure of the protein. Adding  $\beta$ -mercaptopethanol helps to preserve those -SH groups during purification.



**Figure 10.** Native Purification of 6xHis protein in the presence of  $\beta$ -mercaptopethanol. N-terminal 6xHis-tagged mouse DHFR (19.5 kDa) was expressed in *E. coli*. 2 ml of lysate was purified using gravity flow on TALON resin in increasing concentrations of  $\beta$ -mercaptopethanol. Even lanes: 20  $\mu$ l of non-adsorbed material. Odd lanes: 5  $\mu$ l of eluate.



**Figure 11.** Yields of purification in the presence of  $\beta$ -mercaptopethanol compared to Ni-NTA resin. N-terminal 6xHis DHFR was expressed and purified under native conditions. Protein concentrations were determined by Bradford assay. Yields are expressed as a percentage of total protein in the cell lysate.

## Formats of TALON™ Resin

### Physicochemical properties

**Table II: Physicochemical properties of TALON™ Resins**

Features	TALON™ Superflow TALON™ Resin	TALON™ Resin	TALONspin™ CellThru	Columns
Batch/gravity flow applications	Yes	Yes	Yes	No
FPLC applications	No	Yes	Yes	No
Scale	Analytical, preparative, production	Analytical, preparative, production	Preparative, production	Analytical
Capacity (mg protein/ml adsorbent)	5–10	5–8	5–10	2–4
Matrix	sepharose 6B-CL (6% cross-linked agarose)	Superflow (6% cross-linked agarose)	Uniflow (4% cross-linked agarose)	Silica
Bead size (μm)	45–165	60–160	300–500	16–24
Maximum linear flow rate (cm/hr)*	30	3,000	800	n/a
Maximum volumetric flow rate (ml/min)*	0.5	50	13	n/a
Recommended volumetric flow rate (ml/min)	0.3	1.0–5.0	1.0–5.0	0.3
Maximum pressure	2.8 psi 0.2 bar 0.02 MPa	150 psi 10 bar 0.97 MPa	9 psi 0.62 bar 0.02 MPa	n/a
pH stability (duration)	2–14 (2 hr) 3–14 (24 hr)	2–14 (2 hr) 3–14 (24 hr)	2–14 (2 hr) 3–14 (24 hr)	2–8.5 (2 hr) 2–7.5 (24 hr)
Protein exclusion limit (Da)	$4 \times 10^7$	$4 \times 10^6$	$2 \times 10^7$	n/a

\*For washing and elution only.

# Protein Purification Procedures

## Batch

In batch purification, the sample is applied to a tube containing resin. After incubation, the tube is centrifuged and the supernatant is discarded. The resin is washed with buffer and centrifuged. Then, elution buffer is added and the supernatant is collected after centrifugation.

## Batch/gravity flow

Batch/gravity-flow purification means the protein is bound to the resin in solution and then the protein-resin mixture is applied to a column for washing and elution. This procedure gives efficient binding of 6xHis-tagged proteins, most notably when the 6xHis tag is not completely accessible or when the desired protein in the lysate is present in low concentration. By taking this approach, you optimize the time of contact between the resin and your sample. This method is also simpler and requires less equipment than other methods. Batch/gravity flow is usually intended for small-scale purification.

## Standard column chromatography

In column purification, the protein binds the resin directly in the column, not in solution as with batch and gravity-flow purification. The resin is first packed into the column and equilibrated with lysis buffer. Then, the cell lysate is applied to the column. Washing and elution steps follow just as in the batch purification procedure. This method affords higher purity of the final product and is also faster than other methods.

## FPLC (Fast Protein Liquid Chromatography)

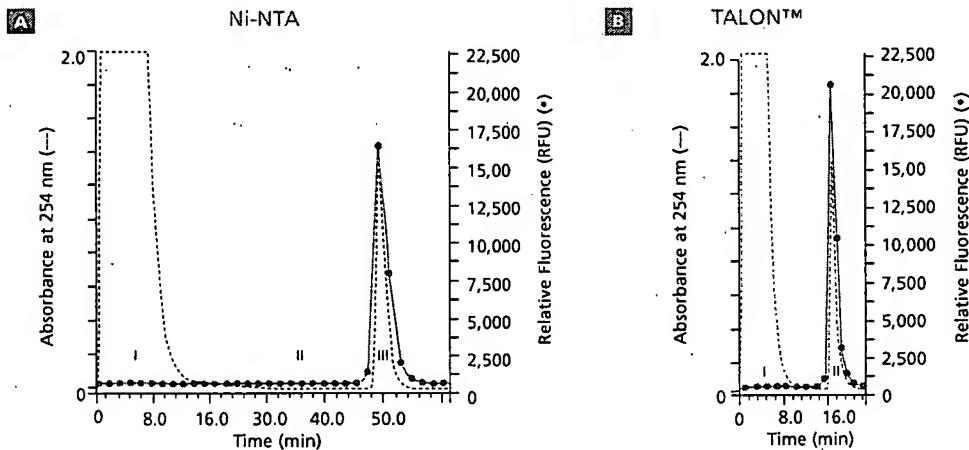
FPLC is a protein purification technique utilizing inert materials, such as glass or plastic, to purify proteins without any metal leaching from the instruments into the protein sample. This method permits you to run chromatography purification at flow rates of 10 ml/min/cm<sup>2</sup> under medium pressure (up to 3 MPa). High flow rates are desirable because you obtain purified protein much more quickly. Fast purification limits the amount of time your protein spends in the presence of proteases (and other impurities) so you get a higher yield of purified product. However, in order to use such high flow rates, the resin must be able to withstand the associated pressure and maintain permeability. TALON Superflow Resin contains specially cross-linked agarose beads that are stronger than conventional agarose beads, so they can be used in FPLC applications. In addition, TALON Superflow beads have high permeability which results in decreased back pressure at elevated flowrates.

## Spin column

Spin-column purification is intended for very small-scale, analytical-grade protein purification. This method is employed when purifying only small amounts of protein from many different samples.

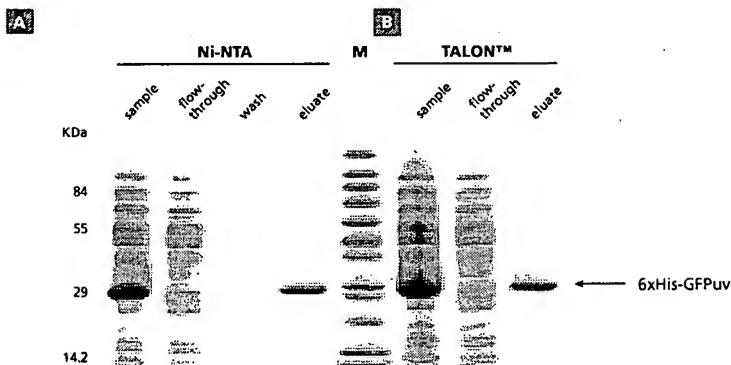
## Purification using FPLC

TALON™ Resin is available in the TALON™ Superflow format, which is useful for a variety of applications, including medium-pressure applications with FPLC systems at back pressures of up to 150 psi (1 MPa). TALON Superflow can be used at high linear flow rates—up to 5 ml/min/cm<sup>2</sup>. This resin is recommended if short purification times are essential, or if purification protocols developed for small or medium scale volumes need to be scaled up for larger volumes.



**Figure 12. FPLC purification of 6xHis-GFPuv with TALON™ Superflow.** Nickel-NTA (Panel A) requires longer washing and lower flow rates to purify 6xHis-GFPuv than TALON Superflow (Panel B). Protein was extracted in 50 mM sodium phosphate, 0.3 M NaCl, pH 7.0. Panel A. 3.2 ml culture filtrate was loaded at 0.5 ml/min. Then nonadsorbed material was washed in the same buffer with 10 mM imidazole. Protein was eluted with 20 mM imidazole (peak II) and 250 mM imidazole (peak III). Panel B. 3.2 ml culture filtrate was loaded at 1 ml/min. Then, nonadsorbed material was washed with the same extraction buffer and eluted with 150 mM imidazole (peak II).

## Purification using FPLC...cont.



**Figure 13. SDS-PAGE of FPLC fractions from 6xHis-GFPuv purification.** FPLC purification fractions from the peaks in Figure 11. Purification with TALON Superflow requires less washing with exceptional results.

Table III: TALON™-compatible reagents	
Reagent	Acceptable concentration
β-Mercaptoethanol	10 mM (with caution)
CHAPS	1% (with caution)
Ethanol	30% (only for storage)
HEPES	50 mM
Glycerol	20%
Guanidinium-HCl	6 M
Imidazole	200 mM at pH 7.0–8.0 for elution
KCl	500 mM
MOPS	50 mM
NaCl	1.0 M
NP-40	1%
SDS	1% (with caution)
Tris	50 mM
Urea	8 M

Incompatible reagents
• DTT (dithiothreitol)
• DTE (dithioerythritol)
• EDTA (ethylenediaminetetraacetic acid)
• EGTA (ethylene glycol-bis(β-aminoethyl ether))

## Purification from Crude Cell Lysates

TALON CellThru is a novel IMAC resin for purifying polyhistidine-tagged proteins from crude cell lysates, sonicates, and fermentation liquids. The large bead size of TALON CellThru (300–500 µm) permits cellular debris to flow through the column, eliminating the need for high-speed centrifugation. Additionally, destabilizing factors are removed more quickly with TALON CellThru than with other resins, because the number of steps is reduced.

### Advantages of direct capture

Traditionally, obtaining protein from crude cell lysates, such as cell culture and fermentation harvests, requires two steps: isolation, followed by column or batch purification. In the isolation step, the removal of particulate material by centrifugation and/or microfiltration is followed by an initial volume reduction step (typically ultrafiltration). Since conventional chromatography columns are quickly clogged by particles such as cells, cell debris, precipitated proteins, the lysate must be particle-free prior to purification. Therefore, the load must be cleaned before applying it to the column.

However, these centrifugation and filtration steps can be time-consuming and expensive and can also compromise quality. Proteases and glycosidases released from the lysed cells can degrade the target protein, complicate purification, and increase purification costs. The longer the target protein is in the presence of the cell lysate, the more likely it is to be degraded.

One alternative to centrifugation and filtration before loading is a technique called direct capture. With direct capture, you can minimize protein degradation, improve product quality and yield, and save time and money. Also, the initial recovery procedure can be simplified if protein capture and debris removal are combined into a single operation. TALON CellThru allows you to purify His-tagged protein directly from crude cell lysates, including serum, tissue extracts, cell culture harvests, fermentation broth and other crude samples on resin-packed, standard low-pressure columns.

A large agarose bead adsorbent is packed into standard chromatography columns whose end-plate frits (filters) have large pores (190 µm) to prevent column blockage. Because of the large bead sizes, particulate material flows between the beads while the soluble product binds to the immobilized metal ions on TALON Resin. Residual particulate material can be removed from the column by using bidirectional high-speed wash pulses. The product is eluted by normal elution methods.

### Expanded bed chromatography vs. top-loading

TALON CellThru can be used in expanded bed chromatography. With this type of chromatography, the crude lysate is applied to the column in an upward rather than downward direction, resulting in increased distance between resin particles (Anspach, *et al.*, 1999). Using the upward flow, the bed does not become clogged and a greater amount of protein is recovered.

Expanded bed chromatography integrates solid-liquid separation, volume reduction, and partial purification all into one step. The amount of cellular debris can be reduced up to five orders of magnitude. The combination of increased distance between particles and the large bead size of TALON CellThru allows for excellent protein adsorption without clogging the bed.

## Purify Protein from Crude Cell Lysates...cont.

The yield from a particular expanded bed or CellThru application depends to a large extent on the efficiency of the extraction procedure in promoting interaction of the target proteins with the resin beads. Incomplete lysis will result in perceived losses of the target protein in the cell debris, which is removed by centrifugation.

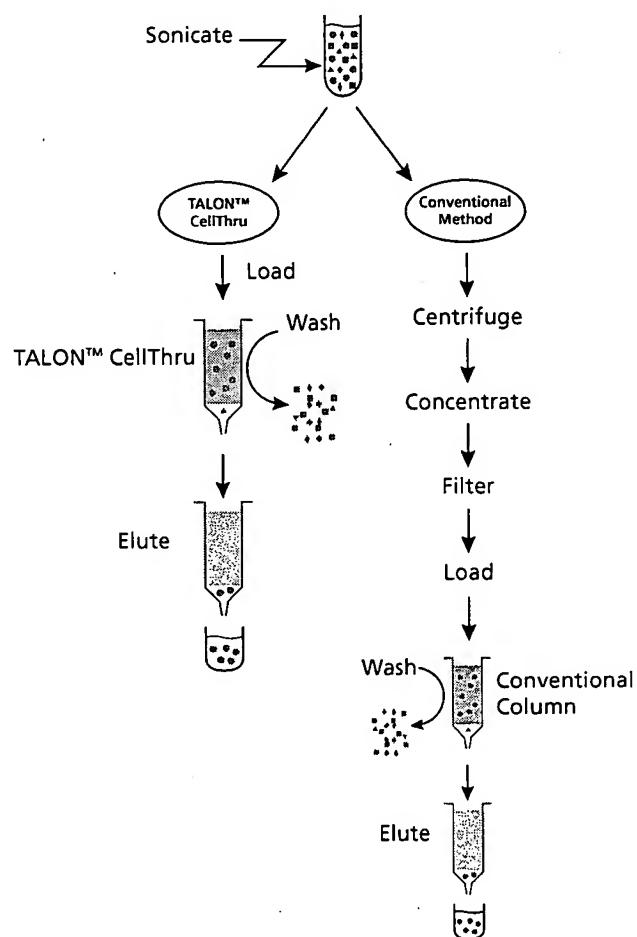


Figure 14. TALON™ CellThru purifies protein from crude cell lysate faster than conventional methods.

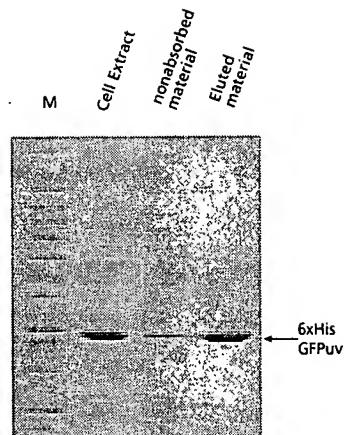
## Purification from Crude Cell Lysates...cont.

CellThru purifies membrane bound proteins and multiprotein complexes

Some proteins are not as easy to access as soluble cytosolic proteins. For example, some recombinant proteins may interact with proteins embedded in the cell membrane (membrane-bound or membrane-associated), while others may be compartmentalized within subcellular organelles. When performing SDS-PAGE analysis, this is generally not apparent because the high SDS and salt concentrations in the sample buffer help solubilize the membranes. Thus, nearly all the proteins present in a cell lysate can be visualized when run on an SDS-PAGE gel.

Purifying membrane-associated proteins with standard TALON Resin is challenging because lysates must be clarified before application to the column. This centrifugation step will usually remove most of the membrane-associated proteins along with the cell membranes and subcellular organelles.

In contrast, with TALON CellThru Resin you can run the crude lysate on the column without centrifuging (direct capture). In this procedure all membranes and unbroken subcellular compartments pass through the column increasing the likelihood of capturing membrane-associated proteins. Therefore, when purifying multiprotein complexes or membrane-associated proteins, TALON CellThru Resin will provide better yields than conventional TALON. However, if a recombinant protein strongly interacts with the membrane or is contained within unbroken subcellular compartments, some proportion of the protein will not be adsorbed by TALON CellThru and will pass through in the wash fractions.



**Figure 15. SDS-PAGE of TALON™ CellThru™ purified proteins.** *E. coli* BL21 cells were sonicated in TALON wash buffer and run through a TALON CellThru column eluted in 150 mM imidazole. Note that some target protein is trapped in membrane fractions and does not get absorbed on the column. M=molecular weight standards.

TALON Product List	Size	Cat. #
TALON Metal Affinity Resin	10 ml	8901-1
	25 ml	8901-2
	100 ml	8901-3
	250 ml	8901-4
TALONspin Columns	10 cols.	8902-1
	25 cols.	8902-2
	50 cols.	8902-3
	100 cols.	8902-4
TALON 2-ml Disposable Gravity Column		
	50 cols.	8903-1
TALON Superflow Metal Affinity Resin		
	.25 ml	8908-1
	100 ml	8908-2
TALON CellThru	10 ml	8910-1
	100 ml	8910-2
CellThru 2-ml Disposable Columns		
	50 columns	8914-1
CellThru 10-ml Disposable Columns		
	20 columns	8915-1
TALON Buffer Kit	each	K1252-1
TALON Purification Kit	each	K1253-1
Talon-Dextran Trial Size	5 mg	8918-y

#### Thiophilic Resin Product List

Thiophilic-Uniflow Resin	10 ml	8913-1
	100 ml	8913-2
Thiophilic-Superflow Resin	10 ml	8917-1
	100 ml	8917-2

#### Glutathione Resin Product List

Glutathione-Superflow Resin	10 ml	8911-1
	100 ml	8911-2
Glutathione-Uniflow Resin	10 ml	8912-1
	100 ml	8912-2
GST Purification Kit	5 purifications	K1251-1

## **Appendix B**

## CELL BIOLOGY & MOLECULAR GENETICS

# Mapping of QTL for Embryo Size in Rice

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## ► ABSTRACT

The development of molecular genetic maps has accelerated the identification and mapping of genomic regions controlling quantitative trait loci (QTL) in rice (*Oryza sativa* L.). Minimizing embryo size in rice would increase proportion of edible endosperm. This study was conducted to determine the genetic basis controlling embryo size of rice grains in a recombinant inbred (RI) population derived from cross of a japonica cultivar, Asominori, with an indica cultivar, IR24, by means of 289 restriction fragment length polymorphism (RFLP) markers. Two parameters, embryo length and embryo width, which represent embryo size in rice, were estimated for each RI line and their parental varieties. Continuous distributions and transgressive segregations of embryo length and embryo width in rice were observed in the RI population, suggesting that embryo size was quantitative in grains of conventional varieties. Three QTL for embryo length were detected on chromosomes 1, 2, and 3 and explained 17.9, 25.7, and 9.2%, respectively, of the total phenotypic variation. Three QTL for the embryo width were observed on chromosome 2, 8, and 10 and accounted for 13.5, 15.7, and 15.0% of total phenotypic variation, respectively. In addition, alleles with increasing and decreasing effects were detected from the both parents. The results and the tightly linked molecular markers that flank the QTL will be useful in breeding for embryo improvement in rice.

- [TOP](#)
- [ABSTRACT](#)
- ▼ [INTRODUCTION](#)
- ▼ [MATERIALS AND METHODS](#)
- ▼ [RESULTS](#)
- ▼ [DISCUSSION](#)
- ▼ [REFERENCES](#)

**Abbreviations:** CIM, composite interval mapping • cM, centimorgan • QTL, quantitative trait locus • RFLP, restriction fragment length polymorphism • RI, recombination inbred

## ► INTRODUCTION

RICE IS A STAPLE FOOD for more than 50% of the world's population. The grain consists of endosperm and embryo. The endosperm is the major edible part for humankind. In contrast, the embryo, which is easily broken during processing, is usually used for an industrial material. At present, in rice-breeding programs, relatively little attention has been focused on embryo size. However, during the past two decades, breeding efforts to improve nutritional quality in rice has focused on enlarging the embryo for industrial purposes. Relative to other parts of the rice grain, the embryo has high concentrations of protein, oil, and vitamins (Juliano, 1985; Koh et al., 1994).

- [TOP](#)
- [ABSTRACT](#)
- [INTRODUCTION](#)
- ▼ [MATERIALS AND METHODS](#)
- ▼ [RESULTS](#)
- ▼ [DISCUSSION](#)
- ▼ [REFERENCES](#)

Satoh and Omura (1981) first found a giant-embryo mutant from a Japanese cultivar, Kinmaze, and mutant gene was located on chromosome 7 (Satoh and Iwata, 1990). In addition, Kim et al. (1991)( 1992) detected three giant-embryo

mutants ( $ge^m$ ,  $ge$ ,  $ge^s$ ), all at the same locus reported by Satoh and Iwata (1990). More recently, Koh et al. (1996) precisely mapped the  $ge^s$  locus controlling super-giant embryo to chromosome 7 using RFLP and microsatellite markers.

If the objectives of breeding programs are to increase embryo size, it is clearly important to study the giant-embryo mutants in rice. On the other hand, if the ultimate goal of a breeding program is to increase endosperm components, it is valuable to minimize the embryo size. Thus, an understanding the genetic basis underlying the inheritance of embryo size in rice has significant implications for quality improvement.

The recent advances in high-density marker linkage maps in rice have provided powerful tools for elucidating the genetic basis of quantitatively inherited traits (Causse et al., 1994; Harushima et al., 1998). As a result, numerous QTL (Yano and Sasaki, 1997) associated with yield and its components and other agronomic traits in rice have been identified and mapped by means of molecular markers. However, to our knowledge, genetic analysis of QTL associated with embryo size has not been conducted in normal grain rice. The aims of this study are to identify QTL for embryo size by means of RI lines from a japonica/indica cross and to determine the relationships between QTL for embryo size and QTL for grain size, which were found in previous studies (Redona and Mackill, 1998; Tan et al., 2000).

## MATERIALS AND METHODS

### Plant Materials

The RI lines in this study, kindly provided by professor A. Yoshimura of Agricultural faculty of Kyushu University, Japan, were developed by single seed descent from the progeny of a cross of japonica cultivar, Asominori, with indica cultivar, IR24. 165  $F_6$  lines were randomly selected from 227 original  $F_2$  individual plants and used for mapping. The RFLP map covering 1275 centimorgans (cM) was constructed with 375 markers from the  $F_6$  and  $F_7$  generations of 71 RI lines (Tsunematsu et al., 1996). In the past, the RI population was used successfully for mapping QTL for important agronomic traits (Yoshimura et al., 1998; Yamazaki et al., 1999; Yamazaki et al., 2000; Sasahara et al., 1999). In this study, we used a subset of 289 RFLP markers, without overlapping, for all loci from the original genetic map (Tsunematsu et al., 1996) to map QTL affecting embryo size in rice, for which the average interval distance between pairs of markers was 4.4 cM.

- ▲ [TOP](#)
- ▲ [ABSTRACT](#)
- ▲ [INTRODUCTION](#)
- [MATERIALS AND METHODS](#)
- ▼ [RESULTS](#)
- ▼ [DISCUSSION](#)
- ▼ [REFERENCES](#)

### Measurements of Length and Width of Embryo in Brown Rice Grains

The RI population along with its parents, Asominori and IR24, were grown at the Experimental Station of Miyazaki University, Japan, during summer-autumn 2001 with two replications in accordance with conventional methods. At maturity, rice grains from each line were harvested and dried naturally in the glasshouse. Then 50 grains from each line were randomly selected and hulled by hand with care and embryo length and width of was measured with a microscope under a magnification of 50x. All measurements for each line were replicated three times. Average values for each line were used for statistical analyses.

### Detection of QTL

Two methods were used simultaneously to identify significant marker locus-trait associations: simple linear regression (single marker analysis) and composite interval mapping (CIM) analysis. The CIM analysis was applied to trait average and marker data to identify more precisely the QTL locations (Zeng, 1994). Single marker analysis and CIM analysis were performed by QTL Cartographer computer program software (Wang et al., 1999) version 1.13 g. The linkages between respective marker loci and putative QTL were determined by single marker analysis. When  $F$  values exceeded a

value necessary for a probability value less than 0.005, the QTL were considered to be significant. CIM analyses were calculated by forward regression, the walk speed of 2 cM, and the window size of 10 cM. A locus with a LOD threshold value of more than 2.5 was to be declared a putative QTL.

In this study, only the QTL detected by both methods were listed. In addition, the additive effect and percentage of variation explained by an individual QTL were also estimated. The QTL were named according to the suggestions of McCouch et al. (1997).

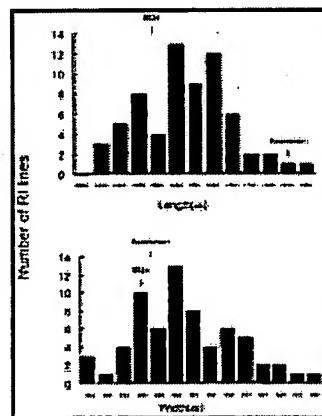
## ► RESULTS

### Distribution of Length and Width in Embryo in Segregating RI Population

The average length and width for embryo of both parents (Asominori, IR24) and the frequency distributions of RI lines are presented in Fig. 1. The two parents, show differences in embryo size, especially for embryo length. Continuous phenotypic variation of both embryo length and width and transgressive segregation suggested that embryo length and width were quantitative traits.

- ▲ [TOP](#)
- ▲ [ABSTRACT](#)
- ▲ [INTRODUCTION](#)
- ▲ [MATERIALS AND METHODS](#)
- [RESULTS](#)
- ▼ [DISCUSSION](#)
- ▼ [REFERENCES](#)

Fig. 1. Frequency distributions for length and width of embryo in rice grains using RI lines derived from a cross between Asominori and IR24.



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### Mapping QTL for Embryo Size Embryo Length

Three QTL controlling the embryo length were identified and mapped to chromosomes 1, 2, and 3 (Table 1 and Fig. 2) and tentatively named for *qEML-1*, *qEML-2*, and *qEML-3*, respectively. The *qEML-2*, located near C132 markers on chromosome 2, showed the largest effect on the trait with a LOD value of 4.3 and explained 25.7% of the total phenotype variation. Another QTL, *qEML-1* (LOD = 3.7), was detected near XNpb393 on chromosome 1 and accounted for 17.9% of total variation. The remaining QTL, *qEML-3*, located near R1468B on chromosome 3, with a LOD value of 2.8, explained 9.2% of total phenotype variation. In addition, the IR24 alleles in both *qEML-2* and *qEML-3* contributed to the decrease in embryo length, whereas *qEML-1* locus increased the embryo length.

**View this table:** Table 1. QTL controlling embryo size in rice based on composite interval mapping (CIM) and [in this window] single marker analysis methods (Wang et al., 1999) using RI lines derived from a cross [in a new window] between Asominori and IR24.

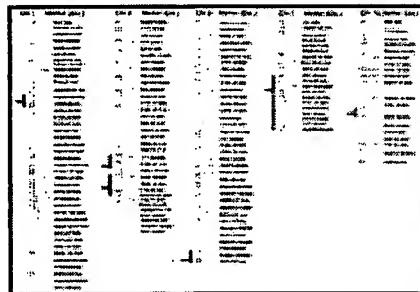


Fig. 2. Chromosomal locations of QTL for embryo size in RI population derived from the cross between Asominori and IR24. Black and shaded bars indicate the genomic regions with  $P < 0.01$  of QTL detected for embryo length and embryo width, respectively, based on single marker analysis. Black arrowheads indicate the location of peak LOD for QTL detected.

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#### Embryo Width

Three QTL (Table 1 and Fig. 2) affecting the embryo width were detected and mapped to chromosomes 2, 8, and 10, and tentatively designated as *qEMW-2*, *qEMW-8*, and *qEMW-10*, respectively. *qEMW-8* had the largest effect (LOD = 5.1) and was located near XNpb41 on chromosome 8 and explained 15.7% of total phenotype variation. *qEMW-2* had a LOD value of 2.6, was located near R712 on chromosome 2, and explained 13.5% of total variation; and *qEMW-10* was located near C1361 marker on chromosome 10 and accounted for 15.0% of total phenotype variation. Further, the two alleles from the indica parent, IR24, in *qEMW-8* and *qEMW-10* decreased the embryo width, while *qEMW-2* allele increased the embryo width.

## ► DISCUSSION

In this study, we report the results of QTL mapping for embryo size with 289 RFLP markers in conventional cultivars using the RI lines derived from japonica Asominori and indica IR24. In the past, the set of RI lines used in our study was used to analyze QTL for days to heading (Yoshimura et al., 1998), whitebacked planthopper (*Sogatella furcifera* Horvath) (Yamazaki et al., 1999), ovicidal response to brown planthopper (*Nilaparvata lugens* Stal) (Yamazaki et al., 2000), and vascular bundle system and spike morphology (Sasahara et al., 1999). From those results, it was shown that these RI lines are very useful for identification of QTL in rice.

Three QTL (*qEML-1*, *qEML-2*, *qEML-3*), located on chromosomes 1, 2, and 3, for embryo length and three QTL (*qEMW-2*, *qEMW-8*, *qEMW-10*), located on chromosome 2, 8, 10, for embryo width were detected. In addition, alleles with increasing and decreasing effects for embryo size were detected from the both parents. The indica parent, IR24, had decreasing alleles for embryo size at *qEML-2*, *qEML-3*, and *qEMW-2*, but increasing alleles at *qEML-1*, *qEMW-8*, and *qEMW-10*, while Asominori alleles had the opposite effects. These results could explain the transgression and continuous distributions for embryo size in the RI population. It is noted that all six QTL detected in the study were independent of the three alleles (*ge<sup>m</sup>*, *ge*, *ge<sup>s</sup>*) that control the giant-embryo trait in rice, located on chromosome 7 in

- ▲ [TOP](#)
- ▲ [ABSTRACT](#)
- ▲ [INTRODUCTION](#)
- ▲ [MATERIALS AND METHODS](#)
- ▲ [RESULTS](#)
- [DISCUSSION](#)
- ▼ [REFERENCES](#)

previous studies (Kim et al., 1991; Satoh and Iwata, 1990; Koh et al., 1996).

However, in an attempt to increase the utilization of rice grains by minimizing embryo dimensions in normal grains, it is a prerequisite that there be neither tight linkages nor pleiotropic effects between QTL for embryo size and grain size. In the past, Tan et al. (2000) reported three QTL for grain length, located on chromosomes 3, 6, and 7 and three QTL for grain width on chromosomes 1, 5, and 8, respectively. Redona and Mackill (1998) also identified seven QTL for grain length, located on chromosomes 2, 3 (two regions), 4 (two regions), 7, 10, and four QTL for grain width, located on chromosomes 2, 3, 7, 8, using an  $F_2$  population derived from tropical japonica/indica cross. In comparing the genomic positions of the QTL identified by Tan et al. (2000) and Redona and Mackill (1998) for grain size with the six QTL detected in our studies for embryo size, *qEMW-8* on chromosome 8 is tightly linked to or allelic to the one for grain width reported by Tan et al. (2000) and Redona and Mackill (1998); furthermore, *qEML-3* on chromosome 3 might be closely linked to a QTL for grain length (Redona and Mackill, 1998), which was obviously different from the one QTL reported by Tan et al. (2000). The other four QTL (*qEML-1*, *qEML-2*, *qEMW-2*, and *qEMW-10*) for embryo size discovered in this research were different from grain-size QTL located in other genomic regions. Hence, these four QTL could be used for rice embryo improvement since they do not affect whole grain size.

In summary, the results from this study can be useful for increasing the utilization of edible endosperms of rice grains by decreasing embryo size in conventional cultivars. The closely linked molecular markers that flank four QTL (*qEML-1*, *qEML-2*, *qEMW-2*, *qEMW-10*) that are independent of grain-size QTL detected in our studies should be very useful for marker-assisted breeding to improve embryo size in rice when transferring the quantitative genes in breeding programs.

## ► ACKNOWLEDGMENTS

We are greatly indebted to Professor A. Yoshimura (plant breeding laboratory, Agricultural faculty of Kyushu University, Japan) for kindly providing materials, molecular data and valuable advice and Professor Phillip E. McClean for his valuable comments in preparation of this paper. This study was jointly supported by a grant from the Heiwa Nakajima Foundation of Japan and a grant from the Iijima Memorial Foundation for the Promotion of Food Science and Technology of Japan. We thank the Japan Society for the Promotion of Science for postdoctoral fellowship (No. P03112) to Dr. Yanjun Dong, which enables him to pursue this study.

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▲ <a href="#">TOP</a>
▲ <a href="#">ABSTRACT</a>
▲ <a href="#">INTRODUCTION</a>
▲ <a href="#">MATERIALS AND METHODS</a>
▲ <a href="#">RESULTS</a>
▲ <a href="#">DISCUSSION</a>
▪ <a href="#">REFERENCES</a>

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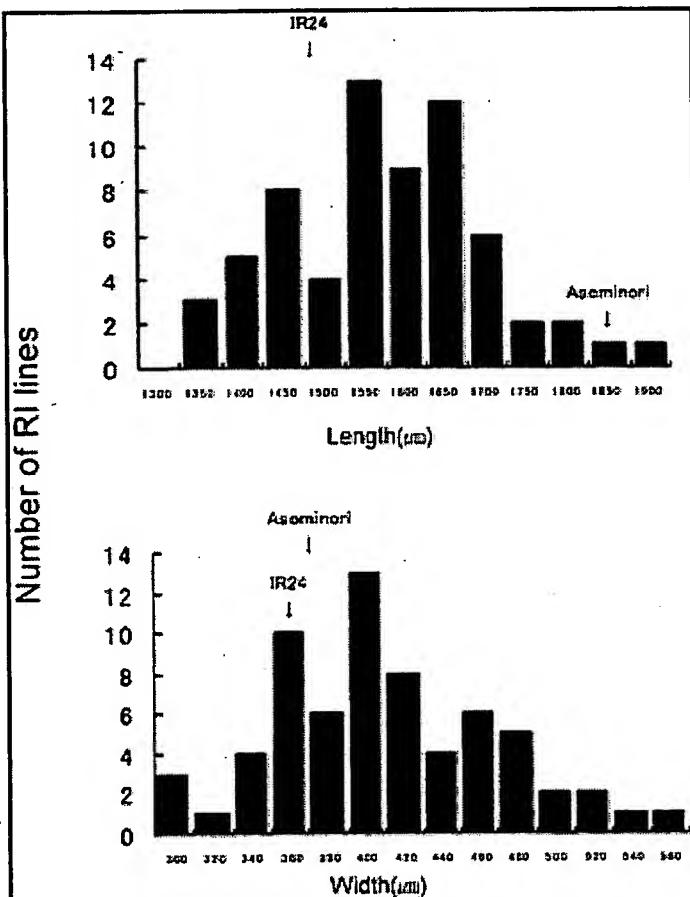
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Fig. 1. Frequency distributions for length and width of embryo in rice grains using RI lines derived from a cross between Asominori and IR24.

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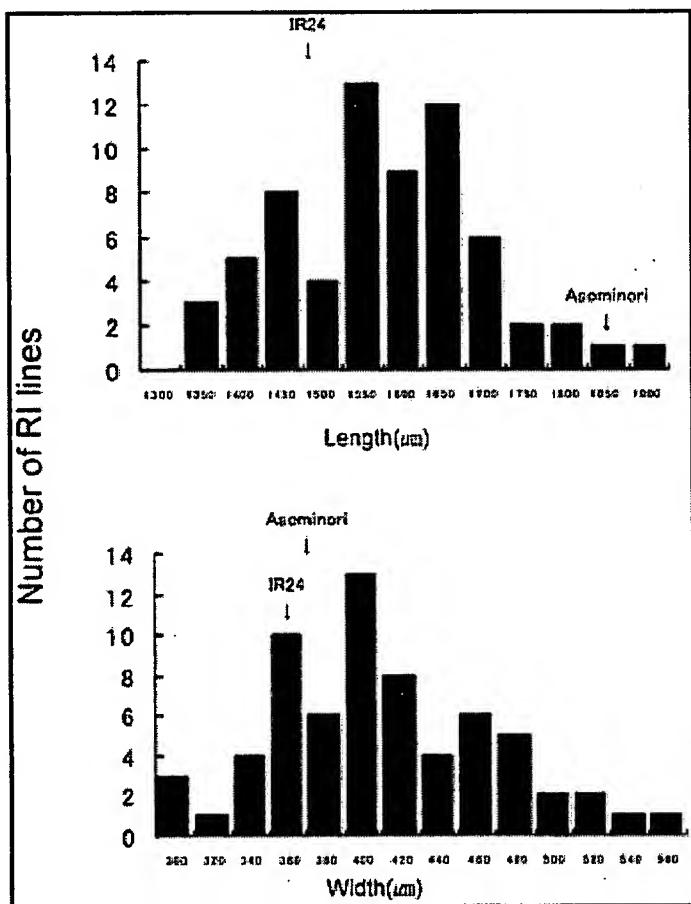


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